Patent
Attorney's Docket No. 0070777-000021

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of	
Tae-Yoon Kim et al.	Group Art Unit: 1656
Application No.: 10/577,775	Examiner: Monshipouri, Maryam
Filed: April 28, 2006	Confirmation No.: 9809
For: EC SOD AND CELL TRANSDUCING EC SOD AND USE THEREOF	

DECLARATION OF DR. Tae-Yoon Kim

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

1. I, Dr. Tae-Yoon Kim, declare the following:

2. I am a citizen of the Republic of Korea, and have the following mailing address: Asia Seonsoochon Apt. #8-702, Jamsil-dong, Songpa-gu, Seoul 138-220, Republic of Korea;

3. I graduated from the Catholic University of Korea, Medical College with M.D. degree in 1980 and Ph.D. degree in 1989;

4. I am a professor of the Department of Dermatology, Catholic University of Korea;

5. I have read and am familiar with the above-identified United States patent application filed April 28, 2006, and I am submitting this Declaration in support of that application;

6. I have performed and/or supervised the experiments reported below:

<Methods>

(1) Cell culture

The cells (HaCaT, A375, and A431) were grown as monolayer cultures in DMEM supplemented with 10% FBS, 100 units/mL penicillin, and 100 μg/mL streptomycin at 37 °C in a humidified 95%-5% (v/v) mixture of air and CO_2. The cells

were then seeded at a rate of 1×10^5 cells per 100-mm plate. After 24 h, they were washed with serum-free medium and placed in serum-free medium prior to the experiments.

(2) Normal keratinocyte culture

Primary cell cultures of normal human epidermal keratinocytes were obtained from Welskin (Seoul, Korea) and maintained in keratinocyte basal medium (KBM Clonetics Corp., San Diego, CA, USA) containing 5×10^{-7} M hydrocortisone, 5 ng/mL of an epidermal growth factor, 30 μg/mL bovine pituitary extract, 5 μg/mL insulin, 50 μg/mL gentamicin, and 50 ng/mL amphotericin B. The medium was changed 2–3 times, and progressively larger keratinocyte colonies were subsequently observed. The cells were starved by culturing them for 24 h in supplement-free KBM that lacked insulin before IGF-II was added to the medium.

(3) Western blot analysis

The cells were lysed with radioimmunoprecipitation assay (RIPA) buffer (2 mM ethylenediaminetetraacetic acid (EDTA), 137 mM NaCl, 20 mM Tris-HCl (pH 8.0), 1 mM sodium vanadate, 10 mM NaF, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1% Triton X-100, 10% glycerol, and a protease inhibitor cocktail) and harvested immediately. The samples were loaded onto sodium dodecyl sulfate (SDS) polyacrylamide gels for electrophoresis and subsequently transferred onto polyvinylidene fluoride membranes. After they were blocked, the membranes were incubated with specific primary antibodies overnight at 4 °C with gentle agitation. The membranes were washed and incubated with a horseradish peroxidase (HRP)-conjugated secondary antibody for 1 h at room temperature. Bands were detected by using ECL reagents.

(4) Transfection

Transient transfections were performed by using Attractene according to the manufacturer's protocol (Qiagen; Santa Clara, CA, USA). In brief, 5×10^5 cells were plated in a 60-mm dish plate 1 day prior to the transfection and grown to approximately 70% confluence. They were then transfected with plasmid DNA, which

was prepared using Attractene at a concentration of 1 μg/plate. The transfections were allowed to proceed for 24 h, and the transfected cells were then washed with 4 mL of PBS and harvested.

(5) FACS (Fluorescence Activated Cell Sorter)

The cells were harvested and washed with ice-cold phosphate-buffered saline (PBS). The pellets were resuspended in 70% ethanol, and then stored on ice. The cells were then permeabilized in a reagent consisting of 0.05% Triton X-100 and RNaseA (100 lg/ml) in PBS. Fixed cells were stained with a propidium iodide (50 lg/ml), and cell cycle phase distribution was analysed using a Becton Dickinson FACScan flow cytometer.

(6) Scratch-wound assay

The A549-control, A549-SOD3(EC SOD) cells were plated onto six-well plates at a concentration of 5×10^5 cells per well. Cell monolayers were carefully wounded by scratching with a sterile plastic pipette tip. The cells were washed twice with cooled PBS and incubated for 24 h. For each wound, 15 fields of excursion were photographed at 0, 24 h after treatment.

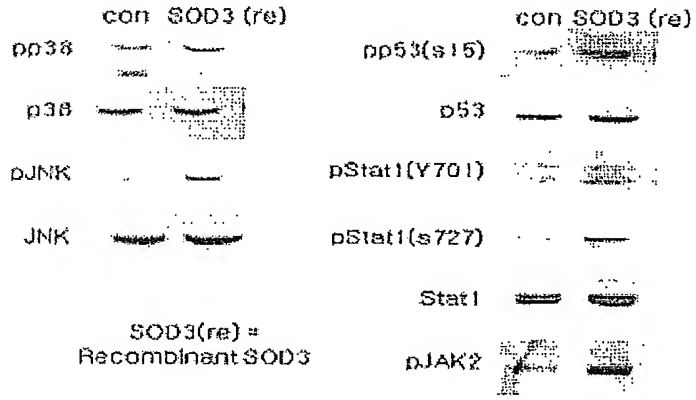
(7) Animal studies

C57BL6 mice were used for all the experiments. The animals were housed and maintained in a barrier facility at the Institute for Animal Studies, School of Medicine, Catholic University of Korea. All the animal protocols used in this study were approved by the Catholic Research Institute of Medical Science Committee for Institutional Animal Care and Use. The mice were maintained under a 12-h light/dark cycle, and they had ad libitum access to food and water. Melanoma cells (5×10^5 cells in 100 μL PBS) and PA (200 μg/mouse on every 4th day) were injected intravenously into the tail vein of the mice. Lung specimens were collected at various time points (8, 12, and 14 day) after injection, and the effects of PA on the growth of metastatic melanoma nodules were investigated.

<Results>

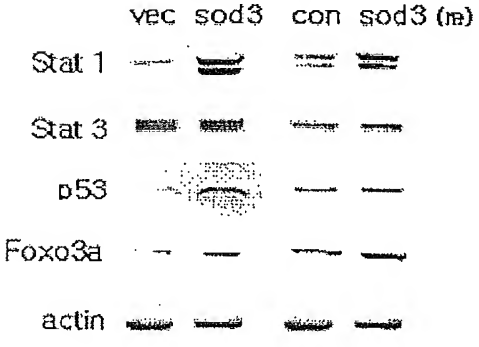
An investigation of whether EC-SOD inhibits proliferation and metastasis in melanoma cells and epithelial carcinoma cells.

<Figure 1> Comparison of activation of cell proliferation regulatory molecules

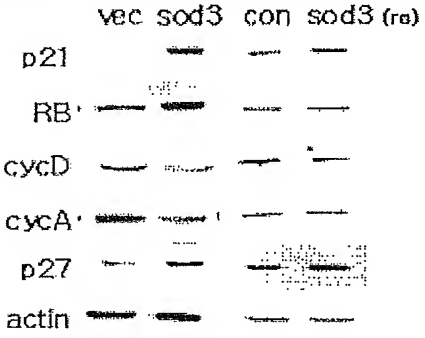


We investigate the effect of EC-SOD on the molecules involved in cell proliferation and apoptosis. Figure 1 shows that EC-SOD induces phosphorylation of p38 and JNK, which inhibit cell proliferation. In addition, we find that p53 and STAT1 are also activated by EC-SOD.

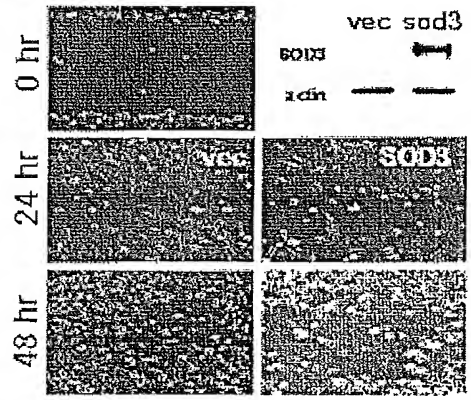
<Figue 2> Comparison of production of cell proliferation regulatory molecules



<Figure 3> Comparison of production of cell cycle regulatory molecules

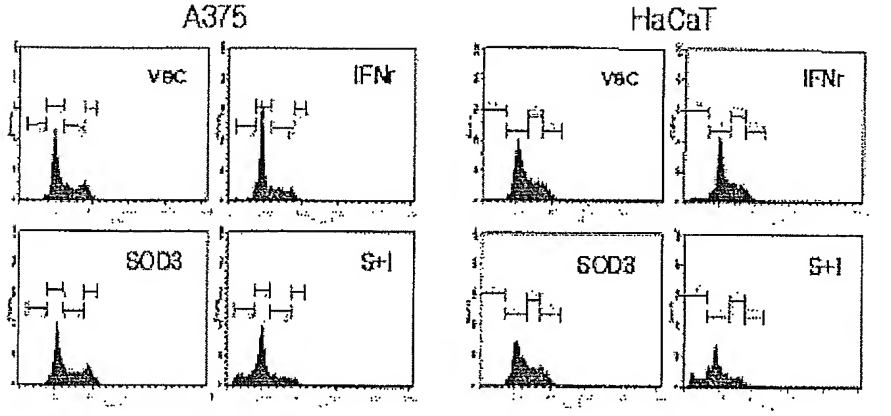


<Figure 4> Inhibitory effect on the cell migration in melanoma cell line that over-express EC-SOD



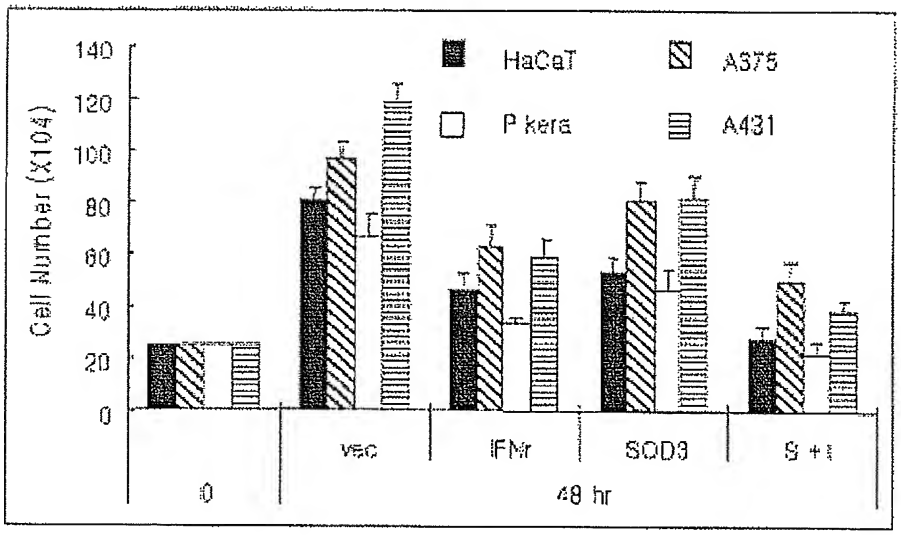
Using the melanoma cell line, in which the EC-SOD gene is over-expressed, it was shown that production of STAT1, p53, and FoxO3a, which inhibit cell proliferation was increased (Figure 2). Furthermore, production of p21, p27, and Rb, which inhibit cell cycle is increased while cycD and cycA, which induce cell cycle, are decreased (Figure 3). It was also determined that EC-SOD effects on the cell migration. The melanoma cells that over-express EC-SOD inhibit cell migration 24h after cell culture (Figure 4).

<Figure 5> Comparison of the effect on the cell cycle and cell proliferation in EC-SOD over-expressed cell lines.



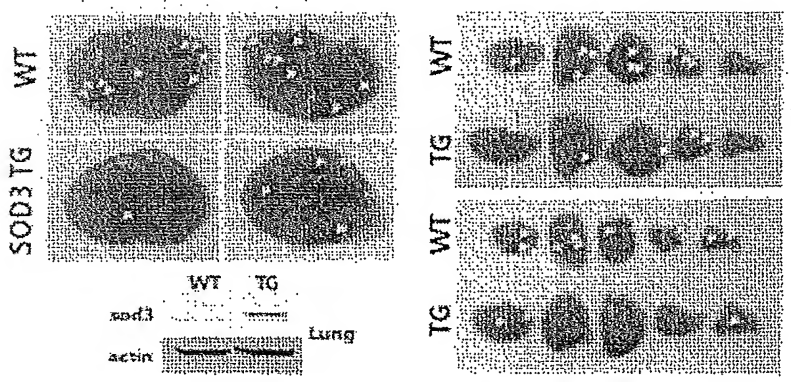
Marker	vec	IFNr	SOD3	S+I (%)
M1 (G1)	61.63	71.51	52.48	59.31
M2 (S)	26.42	20.61	28.87	16.56
M3 (G2)	11.11	3.36	17.35	4.23
M4 (G0)	1.36	5.01	0.82	19.96

Marker	vec	IFNr	SOD3	S+I (%)
M1 (G1)	83.52	66.61	58.55	65.69
M2 (S)	20.13	15.45	23.81	7.37
M3 (G2)	16.24	14.77	17.85	8.31
M4 (G0)	0.82	3.83	0.42	18.35



We determined the effect of EC-SOD on cell cycle and cell proliferation. The results showed that over-expression of EC-SOD results in reduced cell number in the G1 phase. Additionally, IFN-g induced apoptosis increased by approximately 20% when A375 and HaCaT cells were treated with EC-SOD (Figure 5). It was also shown that EC-SOD has a similar effect on another cells, A431, which is human epithelial carcinoma cell line, and primary keratinocytes.

<**Figure 6**> Inhibition of the metastasis of melanoma cells in EC-SOD transgenic mice.



7. Finally, we determined the effect of EC-SOD on the inhibition of metastasis using transgenic mice that over-express EC-SOD. We injected 1×10^6 B16F10 cells, the mouse melanoma cell line, into 6-week-old mice intravenously. On day 14 after injection, the mice were sacrificed, and their lungs were isolated and fixed with 4% formalin for 16 hours. After washing for 16 hours a lung picture was taken and colony numbers were counted. The lungs from transgenic mice showed reduced metastasis of melanoma cells compared to wild type control mice, suggesting that EC-SOD has an inhibitory effect on the metastasis of melanoma cells.

8. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Date: Oct. 21, 2009 By: _____
Tae-Yoon Kim